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TECHNICAL MEMORANDUM 20

RESPONSE OF MONKEYS TO <u>PASTEURELLA</u> <u>TULARENSIS</u>:

HISTOPATHOLOGY OF TULAREMIA INDUCED WITH AEROSOLS OF DIFFERENT PARTICLE SIZE

SEPTEMBER 1962

UNITED STATES ARMY BIOLOGICAL LABORATORIES FORT DETRICK

U.S. ARMY CHEMICAL-BIOLOGICAL-RADIOLOGICAL AGENCY
BIOLOGICAL LABORATORIES
Fort Detrick, Maryland

The work reported here was conducted under Project
4B11-02-068, Aerobiological Research, Task -03,
Pathogenesis of Biological Agents. The expenditure
order number was 2073.

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ABSTRACT

Histopathological responses are described and
evaluated for rhesus monkeys exposed to Pasteurella
tularensis aerosols of different particle size.
The relationship of dosage and particle size in
the disease process is considered.

The primary sites of infection were either the
nasal passage or the lungs. The site was a function
of the concentration and particle size. Larger
numbers of P. tularensis cells were required to
produce an infection as the particle size increased.
Eight to ten microns is the apparent maximum
effective particle size for initiating tularemia
in the lower respiratory tract.

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I. INTRODUCTION

To determine the effectiveness of aerosols of particles larger than one micron in producing tularemia, monkeys were exposed to three separate aerosols. Each aerosol was of a different particle size containing _Pasteurella tularensis_ cells and was administered in four different cell concentrations.

The respiratory system is the portal of entry for many infectious diseases. Infectious particles readily reach the upper respiratory tract and may be retained in sufficient quantity to produce a rhinitis or pharyngitis. In order to reach the distal portions of the lower respiratory tract to produce a pneumonia, infectious material must follow an irregular and gradually narrowing pathway. The size of the infectious particles may, therefore, be a critical factor in the development of a pneumonia or other forms of infectious disease.

Studies have been made with man on these aspects of the problem by investigators in the field of industrial hygiene. They have found that nasal filtration is 90 to 95 per cent efficient for particles larger than five microns;[1-2] its efficiency decreases for particles smaller than that.[3-4] Beyond the nose the depth of penetration increases with decreasing particle size.[5-6] The optimum particle size having the highest probability of alveolar deposition or retention is about one micron.[7-9] "Alveolar retention" includes the surfaces of smaller bronchi and bronchioles in addition to the alveoli in that it all contains "still" air. It is estimated that 45 per cent of the inhaled aerosol in retained.[10]

The diameter of the terminal bronchiole in man has been measured at 350 to 500 microns.[5-11] This measurement in the monkey is approximately the same. A lumen of this size is adequate to receive particles larger than one micron.

II. MATERIALS AND METHODS

The infective organism, the SCHU-D strain of _Pasteurella tularensis_, was disseminated by a spinning disc.[12] The aerosols thus generated were contained in a modified Reyniers chamber[13] at room temperature (20° to 23°C) and at relative humidities of 30 ±5 per cent. The animal hosts were male and female monkeys of the _Macaca mulatta_ species.

The experimental groups included three different particle-size aerosols administered at four different dose levels. The aerosols had particle number median diameters (NMD) of 6.5, 11.5, and 22.0 microns. The NMD is that particle diameter in microns above and below which 50 per cent of the particles are observed to occur. The planned inhaled doses were (a) under 500 cells, (b) under 1000 cells, (c) under 2500 cells, and (d) under 5000 cells. The inhaled doses were controlled by preparing appropriate dilutions of the cultures in modified casein partial hydrolyzate plus variation in the feed rate of the spray menstruum to the disc surface and in animal exposure time. Particle size was controlled by per cent solids, relative humidity, disc speed, feed rate, and temperature. The calculated inhaled doses were determined by sampling the air with glass impingers placed adjacent to the monkeys, then culturing the bacteria. The number of viable cells contained in each aerosol was determined by drawing 9.5 liters of aerosol per minute through 25 milliliters of gelatin saline in a Shipe impinger.[14] Samples of collecting fluid from each animal exposure were assayed for viable organisms by plating on blood glucose cysteine agar.

The actual dose administered to each animal, as calculated by the formulae described by Rosebury,[13] did not always agree with the planned dose.

Each of the three particle-size aerosol groups was composed of 24 monkeys. Each dose group within an aerosol group was to have been composed of six monkeys. The final dose groupings were made on the basis of the actual calculated inhaled dose. This resulted in an irregular dose grouping of the animals in the intermediate particle size aerosol.

Infected control monkeys included: (a) six animals that had 0.2 ml of liquid cultures of _P. tularensis_ introduced into the nasal passages while they were anesthetized, and (b) six animals that received a similar quantity of culture subcutaneously in the ventral surface of the thigh. These animals were necropsied at time of death or sacrifice.

The _Macaca mulatta_ monkeys had an average weight of three kilograms. They were anesthetized with a combination of sodium ethyl (1-methylbutyl) thiobarbiturate and sodium ethyl (1-methylbutyl) barbiturate* administered intravenously prior to their being placed in the aerosol chamber.

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* Combuthal (Abbott Laboratories).

Clinical observations for evidence of infection included recording the occurrence of anorexia and fever, auscultation of the chest, and palpation of the abdomen for hepato-splenic pain and enlargement. Antibody titrations, counts of total number of white blood cells, and blood cultures were conducted.

Detailed necropsies were performed promptly after death. Tissues of 34 different organs of each animal were examined. Multiple sections through the anterior portion of the head were prepared to permit microscopic examination of the tissues of the nasal passages. Neutral formalin was used as a fixative and the sections of tissue were stained with Giemsa stain. The bones in the sections of the nasal passages of animals exposed to the 6.5-micron aerosol were decalcified in sulfuric acid and those of the other two groups were decalcified in formic acid.

III. RESULTS

The monkeys that received the three different aerosols were designated (a) the small-particle aerosol group (6.5-micron), (b) the intermediate-particle aerosol group (11.5-micron), and (c) the large particle aerosol group (22-micron). The results of the exposure of these three groups of monkeys in terms of number of days survived post-exposure for each animal are presented by groups. These results are summarized in Tables I and II and in Figures 1, 7, and 12.

A. SMALL-PARTICLE AEROSOL GROUP

One-half of the monkeys exposed to the smallest dose of 240 cells died of tularemia. All of the monkeys in the groups th t received 720 cells and 4416 cells died. At the 2208-cell dose two monkeys survived to the twenty-eighth day post-challenge. Deaths began as early as the sixth day, and more than 50 per cent were dead by the tenth day. The latest death occurred on the seventeenth day (Figure 1).

The small-particle aerosol had a particle NMD of 6.5 microns. 90.5 per cent of the particles were less than 7.6 microns. None of the remaining particles was under 3.8 microns nor over 10.8 microns.

All of the animals in this group became infected as measured by the development of a temperature over 103.5°F, abnormal breath sounds on auscultation, pain on abdominal palpation, loss of appetite, and, in those few cases where the procedures were applied, positive blood cultures and agglutination titers.

All of the monkeys in this group had active lesions of tularemia. They were present in the lung, peribronchial lymph nodes, soft tissues of the nasal passages, liver, spleen, and bone marrow of each of the 19 monkeys dying of the disease. Twelve of these monkeys also had lesions in the adrenals, and one had a necrotic lesion in the pituitary.

The lesions in the lung were severe necrotizing lesions, which extended along the bronchi (Figure 2) and involved entire lobules and, in many instances, entire lobes of the lungs (Figures 3 and 4).

The lesions in the soft tissues of the nasal passages were related to the blood vessels, which contained thrombi (Figure 5). These were present in all of the monkeys that died of tularemia. In a number of cases, a vasculitis with necrosis developed and extended into the adjacent soft tissues. In several of the animals, this process was severe enough to extend to the mucosal surface and damage the overlying mucosa (Figure 6). Three of the cases had lesions in the retropharyngeal and submental lymph nodes.

Figure 1. Aerosol Number Median Diameter, 6.5 microns.

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Figure 2.   Lung with Peribronchial Necrosis.   X60
Armed Forces Institute of Pathology negative.

Figure 3.   Lung with Lobular Involvement.   X50
Armed Forces Institute of Pathology negative.

Figure 4.   Lung with Lobar Involvement.   X5
            Armed Forces Institute of Pathology negative.

Figure 5.   Thrombi in Vessels of Lamina Propria of Nose.   X80
            Armed Forces Institute of Pathology negative.

Figure 6.   Severe Ulcerative Tissue One Side Nasal Septum.   X50
Armed Forces Institute of Pathology negative.

Five animals in this 6.5-micron-particle group did not die of the
disease spontaneously, but were sacrificed and examined one month post-
challenge.   Each of these animals had active tularemia lesions.   Four had
multiple pulmonary lesions and significant agglutination titers, two of
which were 1/640 and two, 1/1280.   No titration was made on the serum of
the fifth animal, but its final white blood count was 20,500.   It had
numerous tularemia lesions in the liver and only a nonspecific focal arte-
ritis of the smaller vessels in the lung.

B.   INTERMEDIATE-PARTICLE AEROSOL GROUP

All of the monkeys exposed to this particle size became ill clinically,
with the possible exception of one monkey.   Seventeen died and six survived.

The intermediate-particle aerosol had a particle NMD of 11.5 microns.
Ninety-two per cent of the particles were smaller than 12.5 microns; none
of the remaining particles was under 7.6 microns nor over 24.9 microns.

The monkeys in this experiment were not divided into equal dose groups
of six animals each.   Six monkeys received the lowest dose of 556 cells,
and three died.   Five of seven monkeys exposed to 1141 cells and six of

eight monkeys exposed to 2745 cells died of tularemia. The one survivor in the latter group was necropsied three weeks post-exposure and had healing pneumonic tularemia lesions. The early death of one animal was attributed to the anesthetic. The three monkeys that received a dose of 29,863 cells died of tularemia (Figure 7).

All of the 17* monkeys in this intermediate-particle aerosol group that died had the systemic form of the disease. Thirteen of the 17 monkeys had extensive, severe, ulcerative and necrotic lesions involving the lamina propria of the nasal passages (Figures 8 and 9). The lung lesions, however, were focal and small (Figure 10). Large focal lung lesions were present in 6 monkeys that died in the acute phase of the disease and in 2 monkeys that were sacrificed at the end of 3 weeks (Figure 11).

Three monkeys had a conjunctivitis attributable to tularemia, two had a tularemic laryngitis, and one had an ulcerative tracheitis.

.Of the six animals that survived, three had resolving tularemia lesions in the lungs. The remaining three monkeys were free of lesions attributable to tularemia, although one had a healed focus in the bone marrow and another had giant reactive follicles in a cervical lymph node.

C. LARGE-PARTICLE AEROSOL GROUP

Eight of 24 monkeys of this group exposed with large-particle aerosols died. The clinical responses of some of the monkeys were slight. The monkeys in the low-dose group did not appear critically ill, although within the first week post-exposure they developed a fever of 103.5° to 104°F, hepato-splenic pain and splenomegaly, and changes in their breath sound. All of the monkeys in the remaining three dose groups did become clinically ill, and their maximum temperature ranged from 104° to 106.5°F.

This large-particle aerosol had a particle NMD of 22.0 microns, and 86.2 per cent of the particles were smaller than 24.9 microns. None of the remaining particles were under 12.5 microns nor over 35 microns.

The animals in the low-dose group (146 cells) did not die during the 40-day observation period. One monkey in the 873-cell group died of tularemia 17 days post-challenge. There were two deaths among the group that received 2315 cells. Four of the six monkeys that received 11,616 cells died of tularemia (Figure 12).   •

Only one of the six animals in the low-dose group was examined by necropsy, and no lesions of active tularemia were found. Tissue changes that could not be positively attributed to tularemia included a healed encapsulated area in the upper lobe of the right lung and eosin staining areas in carinal and peribronchial lymph nodes.

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* One monkey died by anesthesia.

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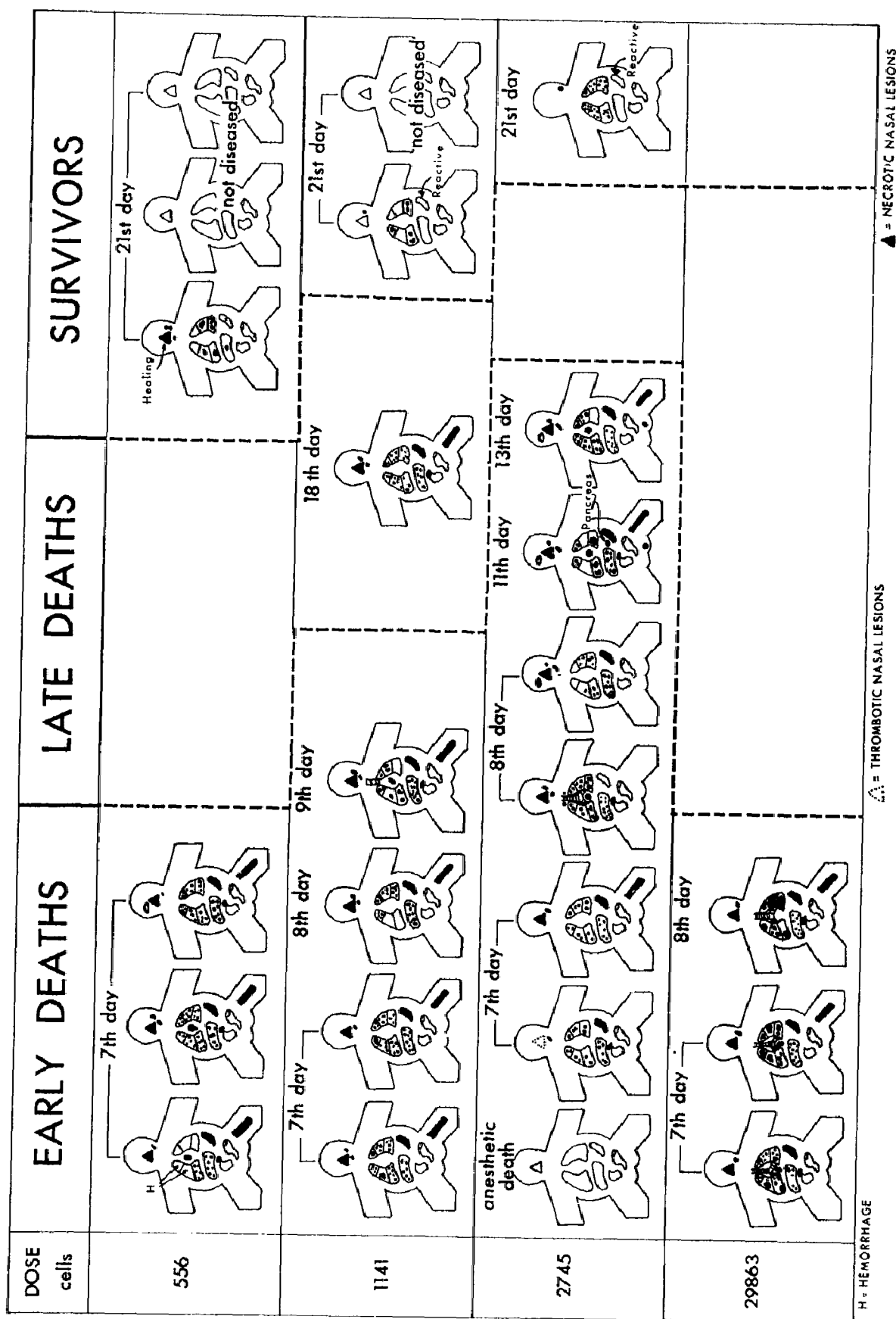
Figure 7. Aerosol Number Median Diameter, 11.5 microns.

Figure 8. Severe Ulceration and Necrosis of Nasal Mucosa (low power). X5
Armed Forces Institute of Pathology negative.

Figure 9. Severe Necrosis, Inferior Region of Nasal Septum (high power).
X50 Armed Forces Institute of Pathology negative.
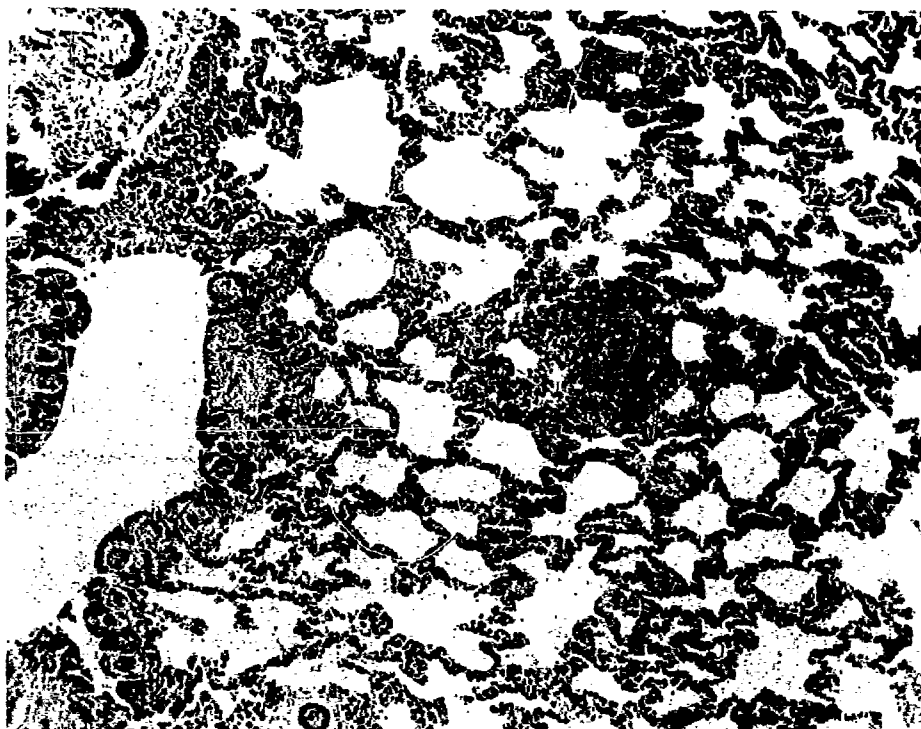
Figure 10. Small Focal Lesions. X80
            Armed Forces Institute of Pathology negative.

Figure 11. Large Focal Lesions. X6
            Armed Forces Institute of Pathology negative.
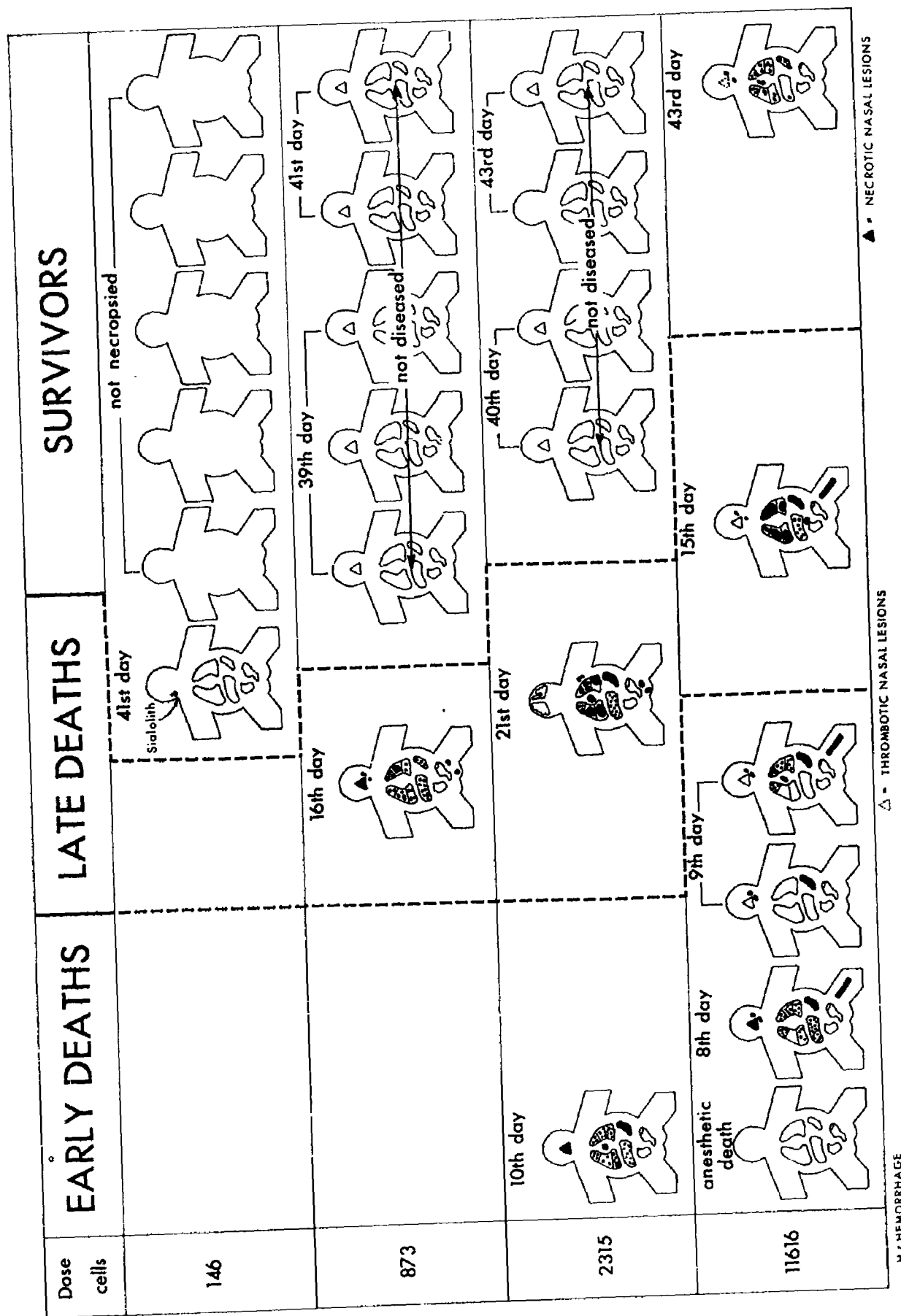
Figure 12.   Aerosol Number Median Diameter, 22.0 microns.

All six of the animals that received 873 cells were necropsied, but only one animal died of tularemia. This animal, which died on the sixteenth day post-challenge, had severe ulcerative tularemia lesions of the mucosa of the nasal passages and necrotic lesions in a retropharyngeal lymph node. Multiple focal lesions were present in the spleen, liver, and lungs; the only pulmonary lesion of moderate size was in the left middle lobe. The five remaining animals were free of lesions of tularemia.

Two of the six monkeys that received 2315 cells died with multiple tularemia lesions. The one that died on the tenth day had severe necrotic lesions in the lamina propria of the nasal passages, with only one large pulmonary lesion in the right lower lobe. The animal that died on the twenty-first day had multiple large lesions of the lungs.

The six monkeys that received 11,616 cells developed tularemia, with one exception. That animal died the day after aerosol exposure, and the death was attributed to the anesthetic. Four of the monkeys died of tularemia. Severe lesions were present in the lamina propria of the nasal passages in the one monkey that died on the eighth day and the two monkeys that died on the ninth day. The monkey that died on the fifteenth day had large pulmonary lesions in addition to the nasal lesions. The surviving monkey was necropsied six weeks post-challenge and had scarring in the lamina propria of the nasal tissues (Figure 13) and small lesions in the lungs, hilar lymph nodes, spleen, and liver.

D. INTRACUTANEOUSLY AND INTRANASALLY EXPOSED CONTROL ANIMALS

The intracutaneously exposed control monkeys developed ulcerative skin lesions and necrotic lesions of the inguinal lymph nodes, spleen, liver, and bone marrow. Minute lesions did not develop in the lungs until the seventh and eighth post-exposure days. Thrombi were numerous in pulmonary blood vessels and in the blood vessels of the tissues of the nasal passages. Animals dying on the fourteenth day had large lung lesions.

The intranasally exposed control monkeys developed ulcerative lesions of the epithelium of nasal passages (Figure 14). These were present six days post-exposure. Lesions of tularemia also developed in the lungs, spleen, liver, and bone marrow. A control monkey that had its conjunctiva washed with a culture of P. tularensis cells developed a severe, exudative conjunctivitis (Figure 15).

Figure 13.   Scarring in Lamina Propria of Monkey Sacrificed
              6 weeks Post-Challenge. X50
              Armed Forces Institute of Pathology negative.

Figure 14.   Severe Ulcerative and Necrotic Rhinitis in Control Monkey
              with P. tularensis Cells Instilled into Nose. X5
              Armed Forces Institute of Pathology negative.

Figure 15. Acute Tularemia Conjunctivitis in the Monkey.
U.S. Army Photograph.

IV. DISCUSSION

The experiments reported here present in terms of deaths, survivals, and pathological tissue changes in monkeys, the results of exposing them to various doses of P. tularensis cells disseminated in aerosols having particle NMD's of 6.5, 11.5, and 22.0 microns.

It is assumed that the development of tularemia in animals exposed to an aerosol of P. tularensis cells occurred because the aerosol entered the respiratory system and the bacteria invaded its tissues. This system consists of an upper portion (the nasal passages, accessory air sinuses, and the pharynx) and a lower portion (the trachea, bronchi, and alveoli). It is obvious that many of the aerosol particles, especially those of larger size, impinge upon and remain on the surfaces of the upper respiratory tract. P. tularensis cells in fine-particle aerosols impinge upon the surfaces of the bronchial tree.

The tularemia-producing effectiveness of aerosols of particle sizes larger than one micron must be due primarily to the ability of the P. tularensis cell to invade the tissues of the respiratory tract at more than one level. The detailed gross and histopathological observations recorded for these experiments provide a means of analyzing the amount and nature of the disease process in the upper and lower portions of the respiratory tract, and of relating them to the dose and particle size of the aerosol.

A. AEROSOL

1. Dosage Variation within Particle-Size Groups

The variations in dose size in the different aerosol groups were not considered detrimental to the experiment.

It is known that fine-particle aerosols are more effective in penetrating and being retained in the smallest air passages of the lung than are coarse-particle aerosols, and so it would be expected that a smaller infective dose of bacterial cells would be required for the fine-particle aerosol than for the ones with larger particles. It was for this reason that four different dose levels were included in each of the three different particle-size aerosols. In order to make a comparison, it was desirable to have each of the doses uniform. Variation in dose did develop in the course of the experiments.

In the 500-cell dose group, 240 cells were inhaled in the 6.5-micron aerosol, 556 cells in the 11.5-micron, and 146 cells in the 22.0-micron aerosol. It would have been preferable to have had 500 cells in the 22.0-micron aerosol rather than 146 cells, but in view of the single death

in this aerosol at the 873-cell dose, it is unlikely that the response of the monkeys to a 500-cell dose would have been significantly different from their response to a 146-cell dose.

The two intermediate-sized doses designed to provide 1000 cells and 2500 cells were uniform for each of the three different sized aerosols.

In the 5000-cell dose group 4416 cells were inhaled in the 6.5-micron aerosol, 29,683 cells in the 11.5-micron aerosol, and 11,616 cells in the 22.0-micron aerosol. The high dose in the 11.5-micron aerosols was not harmful to the experiments, as 1141 cells and 2745 cells had both acted as essentially an LD_{100}. In the 22.0-micron aerosol, 2315 cells produced death in two of six monkeys. It is conceivable that a 5000-cell dose would have produced better than an LD_{50}. The 11,616-cell dose did kill most of the monkeys in the group, so it was established that the large-particle aerosol can kill monkeys with this relatively small dose.

2. Effect of Dosage on Death

The dosage of inhaled organisms is of significant importance. All three particle-size groups produced death, but more organisms were required as the particle size increased.

In the 500-cell dose group, six monkeys died of the disease, four survived with the disease, an additional survivor had a lesion in the bone marrow, and seven survived in whom evidence of the disease was not obtained. Only two of these non-diseased survivors were sacrificed and necropsied, and they were free of active disease.

In the 1000-cell dose group, 12 monkeys died with tularemia, one survived with the disease, and six survived with no evidence of the disease.

In the 2500-cell dose group, 12 monkeys also died of the disease, three survived with tularemia lesions, and four survived with no morphological evidence of the disease. The one animal that died an anesthetic death was not included in the group. In the planned 5000-cell dose group, as previously stated, there was a greater variation in dose than in the other groups; 4416, 29,863, and 11,616 cells were inhaled. Nevertheless, 13 animals in this group survived without evidence of the disease. One animal's death was attributed to the effects of the anesthetic.

This information suggests that, in tularemia, the number of cells contained in the aerosol is important and can improve the effectiveness of large-particle aerosols. No determinations were made of the number of cells in each particle. A 22.0-micron aerosol containing 11,616 cells appeared to be as effective in establishing tularemia in monkeys as were 1141 cells in a 11.5-micron aerosol, or 720 cells in a 6.5-micron aerosol. This does not necessarily contradict the fact that an aerosol of a one- to four-micron particle size would be more effective in establishing tularemia.

The important feature is that 6.5-, 11.5-, and 22.0-micron-particle aerosols are capable of infecting monkeys if the dose is large enough.

3. Effects of Particle Size on Death

The death response of the monkey may also be analyzed solely on the basis of the particle size of the aerosols without indicating the doses; The smaller particles have a greater capacity for producing disease and death.

The aerosols produced by the spinning disc disseminator, although made up of a heterogenous particle distribution, had a heterogeneity that extended only over a very limited size range. The 6.5-, 11.5-, and 22.0-micron aerosols are accepted as independent aerosols, with a negligible degree of overlap.

In the 6.5-micron aerosol, 0.3 per cent of the particles were 3.8 microns in diameter. Only 4.8 per cent of the particles were 5.6 microns in diameter (Figure 16). This places the 6.5-micron aerosol above the 1- to 4- or 5-micron range[1-2] or the range of greatest efficiency for delivering particles to the finest portions of the bronchial tree.[5-8] It is doubtful that the bacteria in this five per cent of the aerosol could have produced the numerous deaths and the multiple lesions observed in the monkeys' lungs.

In the upper range of the 6.5-micron aerosol, 85.4 per cent of the particles were 7.6 microns in diameter, compared with 0.5 per cent of this sized particle in the 11.5-micron aerosol. The next overlap was in the 10.8-micron range, with this particle size accounting for 9.5 per cent of the 6.5-micron aerosol and 7.8 per cent of the 11.5-micron aerosol.

The important aspect of the 11.5-micron aerosol is that the 7.6-micron particle was the smallest particle detected in the aerosol. This particle has approximately twice the diameter of a 4-micron particle, or twice the size of what has been found to be the upper limit in size for particles that passed through the nose.[1]

The 11.5-micron aerosol contained 83.8 per cent 12.5-micron particles, compared with 0.3 per cent of this sized particle in the 22.0-micron aerosol. This was the smallest particle detected in the 22.0-micron aerosol.

It will be noted in Figures 1, 7, and 12 that the number of deaths is essentially the same in both the 6.5- and 11.5-micron groups. In the 22.0-micron group, the deaths are reduced by approximately two-thirds. The total range of the three aerosols combined includes particles with a diameter of 3.8 microns to 35.0 microns. The effective maximum particle size must extend to at least 12.5 microns, as that was the smallest particle detected in the 22.0-micron aerosol. It should, therefore, be reasonable to expect an aerosol composed of particles of a size midway between the smallest particle detected (3.8 microns in the 6.5-micron aerosol) and the 12.5-micron particle to be reasonably effective in establishing primary pulmonary tularemia.
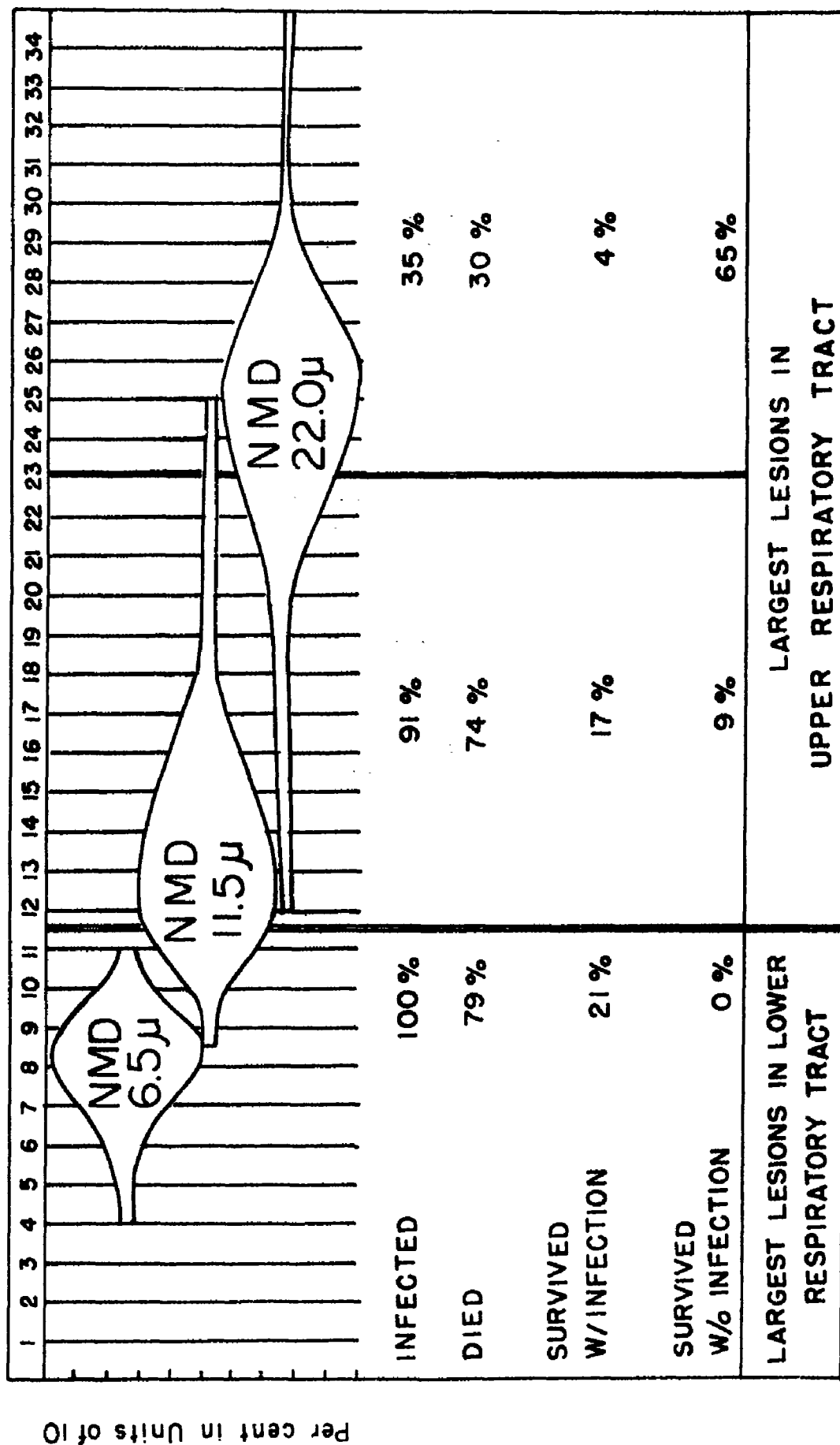
Figure 16. Comparison of the Infectivity, Lesions, and Death of the Aerosol Particle Groups of P. tularensis.

Further studies, therefore, are planned with an eight-micron aerosol. The justification for this is reinforced by the findings of Landahl and Black,[1] which have been referred to previously, as well as those of Davis.[11] Landahl and Black found that eight-micron corn oil droplets could pass through the nose successfully. Davis[11] calculated that drops larger than seven microns would possess enough kinetic energy to traverse distances comparable with the channel width of the maxilloturbinates of the rabbits, so that deposition would be possible where the walls project or curve in any way. He considered that, once past the turbinates, the drops would penetrate into the lung. He, of course, also recognized that drops of 1.5-micron diameter penetrated the nasal channels freely.

B. PATHOLOGY

In the discussion thus far the response of the animals in this experiment has been evaluated principally on the basis of the death pattern, and it has been found that (a) aerosols of 6.5 microns and 11.5 microns particle diameter that contain P. tularensis cells are equally effective in producing disease and death; and (b) the greater the number of P. tularensis cells present, the more effective is the aerosol in producing disease and death, even in an aerosol of 22.0-micron particle diameter. It cannot, however, be concluded from these results that the morphology of the disease in the monkey is similar under the different experimental conditions.

If, therefore, the response of the animals in this experiment is evaluated primarily on the basis of the development of lesions as found through detailed histopathological examination, information is provided on the site and the degree of involvement of different areas of the respiratory tract by the various particle-sized aerosols. An evaluation of this disease pattern as it appeared in the monkeys will be limited to a consideration of the primary site of disease, which in some animals was in the lung and in others the nasal passages. These are first compared with the systemic involvement that follows a primary skin lesion.

1. Pulmonary and Upper Respiratory Tract Pathology in Skin Exposure

Tularemia, as a systemic disease, damages the entire reticuloendothelial system. Lesions develop in lymph nodes, spleen, bone marrow, and liver. Lesions also develop in the lungs and nasal passages in fatal tularemia no matter what the portal of entry. The incubation period in monkeys averages three days, and deaths begin to occur by the sixth or seventh day post-exposure.

In order to evaluate the tularemia lesions in the lung of an animal exposed to a fine-particle aerosol, it is helpful to compare them with lung lesions as they occur in animals with a primary skin lesion. The lung lesions in this latter group result from P. tularensis cells' being transported there by the blood and lymph. Their size, shape, distribution, and time and rate of development are different from those that occur after aerosol exposure.

In the present experiment organisms were introduced into the skin only in the one control group of animals. No primary lesions developed on the cutaneous surface of the aerosol-exposed monkeys, unless the occurrence of a conjunctivitis in two of the monkeys should be accepted as such.

The skin-exposed control monkeys that died on the seventh day post-exposure had lesions in the regional lymph nodes, as well as in the spleen, bone marrow, liver, and the lungs; they had thrombi in the blood vessels of the nose. The lung lesions were minute and involved segments of the alveolar wall or one or two alveoli, as in Figure 10. The control animals that died after the fourteenth post-exposure day had lesions in the same organs. The lung lesions, however, were larger and involved entire lobules and even lobes, and could encompass bronchi. The focal lesions still tended to be rounded and relatively uniformly distributed (Figure 17).
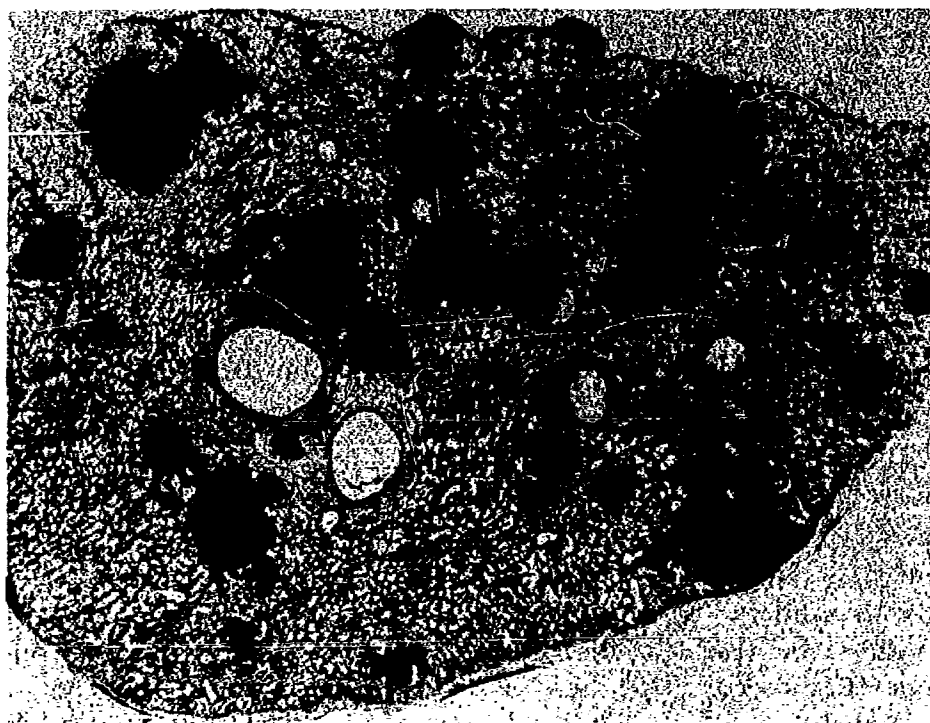

Figure 17. Rounded and Relatively Uniformly Distributed Lung
            Lesions in Skin of Control Monkey. X5
            Armed Forces Institute of Pathology negative.

Although it was possible that some of the P. tularensis cells had been inhaled from the surface of the skin lesion, this possibility was considered remote, because the monkeys were always housed in a room irradiated with ultraviolet light. In skin-injected monkeys, therefore, the small size of the lung lesions at seven days, their rounded shape and uniform distribution, made them acceptable as lesions of hematogenous origin.

If the respiratory system is the portal of entry by exposure with a fine-particle aerosol, then the lungs will contain lesions with a lobular or even lobar distribution in the animals that died on the seventh day. The smaller lesions that may be present are associated with the bronchi and extend to involve adjacent alveoli. Because the lesions develop initially in the lung in animals exposed to fine-particle aerosols, they are larger at the end of seven days than are the metastatic pulmonary lesions that develop following skin injection.

Should lesions develop in the upper respiratory tract and then spread through the blood stream to the lungs, these secondary lung lesions would be small and have the same shape and distribution as in cases of primary cutaneous tularemia.

Monkeys from each of the two different portal-of-entry groups that died after the fourteenth day post-exposure had a mixture of enlarged metastatic tularemia lesions and primary lesions. When the late-death animals were evaluated separately from the early deaths, especially those that received the 22.0-micron aerosol, the significance of the large pulmonary lesions as primary lesions in the 6.5-micron-aerosol tests became apparent.

The evidence in support of these distinctions of primary and secondary lesions, based primarily on the size of the lesions, is analyzed in the following two sections.

2. Primary Lung Pathology with 6.5-Micron Aerosols

Of the 24 animals that received the 6.5-micron aerosol, 16 died by the twelfth day; the majority of these deaths had occurred by the ninth day. Each of the 16 monkeys had large focal pulmonary lesions that extended along the peribronchial tissues and involved entire lobules and even lobes of the lungs (Figure 3). This was in marked contrast to the small secondary lesions in the lungs of cutaneously exposed control monkeys that died within the same period of time. The lesions in the spleen, liver, and bone marrow were essentially similar in both groups of monkeys.

The lamina propria of the nasal passages of the monkeys in the 6.5-micron group contained thrombi within the blood vessels as well as a vasculitis and focal necrotic lesions. The focal lesions were small and were considered secondary to the lung lesions. Small vascular and thrombotic

lesions occurred in cutaneously challenged animals to the same extent that they did in the 6.5-micron-group monkeys that died of the disease. The nasal lesions thus were accepted as due to blood stream dissemination of P. tularensis cells.

The three monkeys in the 6.5-micron group that died on the fifteenth, sixteenth, and seventeenth days post-challenge also had large lung lesions and small nasal lesions. The size of the lung lesions after the second week of the disease may be the same in both cutaneous- and aerosol-challenged monkeys. Their large size was probably due in part to the progressive extension of the disease process over a longer period of time. These delayed-death animals, therefore, were not considered as suitable for distinguishing between cutaneous- and aerosol-exposed animals as were the deaths that occurred 7 to 10, or even 14, days after exposure.

Among the five surviving monkeys, three had larger pulmonary lesions and only one had healing nasal lesions. One of the survivors had only minute, resolving pulmonary lesions, and the other survivor had no pulmonary lesions but did have numerous minute liver lesions.

Both Ohara[15-16] and Ashburn[17] have reported cases of the pneumonic form of the disease in humans, which they attributed to the inhalation of P. tularensis cells.

3. Primary Nasal Pathology with 11.5- and 20.5-Micron Aerosols

Of the 24 monkeys that received the 11.5-micron aerosol, 16 died by the thirteenth day, with the majority of these deaths occurring by the ninth day. Each of the 16 monkeys had extensive necrotic and ulcerative lesions of the lamina propria of the nasal passages. Eleven monkeys had large nasal lesions and discrete small lung lesions comparable to those seen in cutaneously exposed monkeys dying within a similar time period. This suggested that the primary lesion or lesions developed in the nasal passages and, by the time death occurred, produced extensive necrosis in this region. Necrotic lessions were also present in the retropharyngeal and anterior cervical lymph nodes. Only small lesions developed in the lung prior to death, as the P. tularensis cells were carried there in the blood after the nasal lesions had been initiated. These features constituted a major difference between the 6.5-micron group and the 11.5-micron group.

The ulcers, which involved the mucosa of the trachea in several monkeys, were also considered primary lesions. The tracheas were not opened longitudinally in all cases, so the single sections selected were taken in a random fashion rather than an area with a gross lesion having been deliberately selected.

A few lung lesions, similar in size and distribution to those in the 6.5-micron group, were present in the lungs of six monkeys in this group. They occurred at each dose level. All of the six animals had

extensive lesions of the lamina propria of the nasal passages. It is possible, then, that the aerosol was responsible for initiating nasal and pulmonary lesions simultaneously. The control monkeys that had cultures instilled into the nose did develop both nasal and pulmonary lesions. Attempts to expose the epithelial surfaces of the nose only were made by Lewis,* who sought to protect the lungs from being soiled with P. tularensis cells once they were deposited in the nose by maintaining a clear air supply to the lungs through a tracheotomy. This method did not clarify the problem, because of technical difriculties in maintaining the tracheotomy.

Three monkeys in the 11.5-micron group survived 21 days and were then sacrificed and necropsied. Severe nasal lesions were present in one monkey, resolving lesions in another, and no nasal lesions were found in the third monkey. All had local lung lesions, but none were severe.

Of the 24 animals that received the 22.0-micron aerosol, seven monkeys died. The lesions in these animals were similar to those in the 11.5-micron group. The four monkeys that died ten days post-challenge had large nasal lesions, and only three of the monkeys had discrete small lung lesions. The fourth monkey had no lung lesions. These findings were similar to those in the majority of monkeys that received the 11.5-micron aerosol.

The three 22.0-micron-aerosol animals that died on the fifteenth, sixteenth, and twenty-first days had large lung lesions, which were attributed to the natural progression of hematogenous lesions. Five low-dose monkeys were not necropsied. The remaining ten monkeys were necropsied at 41 days. The animal that received 146 cells had sialoliths in a salivary gland. Another necropsied at 43 days post-exposure to 11,616 cells had resolving tularemia lesions in the nose, cervical lymph nodes, lungs, spleen, and liver.

Although the 11.5- and 22.0-micron groups were similar in regard to their pattern of tissue response, they differed in their sensitivity to the size of the doses administered. The 22.0-micron group was infected by the highest dose, but the majority of the low-dose monkeys did not become infected. It may be that, at the lower doses, the number of P. tularensis cells within the 22.0-micron particles may not have been of a concentration great enough to initiate local tissue damage and penetration of the mucosa of the nasopharynx.[18]

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* R. B. Lewis, personal communication.

C. MISCELLANEOUS FACTORS

Various factors that may have influenced the initiation of the disease could include: (a) a break in the surface lining of the nose due to trauma or pre-existing disease; (b) variations in the diameter of the nasal passages due to congenital factors; (c) variations in the rate of respiration among monkeys at the time of inhalation of the aerosol; (d) variations in posture of the monkeys while anesthetized, both during exposure in the chamber and during recovery, as well as other factors. The rather remarkable uniformity of reaction of the two major response groups precludes these factors from having too great a significance.

The studies of Hatch,[9] which indicated that only particles one micron or less in diameter reach the terminal bronchioles, were made with inert particles. He* concedes that a few larger particles may reach the bronchioles, but that this loss of a few large inert particles cannot be detected with the methods he employs.

It is possible, therefore, that a few large infectious particles from aerosols of any size may reach the bronchioles and the living organisms they contain multiply and produce a lesion. The deposition of a few large infectious particles deep within the lung is thus readily manifested, while the loss of a few large inert particles deep within the lung would escape detection. The presence of satellite particles could also provide an explanation.

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* T. Hatch, personal communication.

V. SUMMARY

Pulmonary lesions of large size, associated with the bronchi, develop in the majority of monkeys exposed to P. tularensis cells in aerosols with a particle NMD of 6.5 microns. The portal of entry in these cases is accepted as being the lower respiratory tract.

Nasopharyngeal lesions of large size develop in the majority of monkeys exposed to P. tularensis cells in aerosols with particle NMD's of 11.5 and 22.0 microns. The portal of entry in these cases is accepted as being the upper respiratory tract.

Nasal passage lesions in the majority of the animals exposed to 6.5-micron aerosols were small and are accepted as being due to hematogenous spread.

Pulmonary lesions in the majority of the animals exposed to 11.5- and 22.0-micron aerosols were small and are accepted as being due primarily to hematogenous spread.

Cases with large lesions in both the upper and lower respiratory tract do occur, so that following aerosol exposure, the possibility exists that P. tularensis cells may enter the host simultaneously through these two different portions of the respiratory tract.

Larger numbers of P. tularensis cells are required to produce an infection with aerosols with a particle NMD of 11.5 or 22.0 microns than are required with an aerosol with a particle NMD of 6.5 microns.

Eight to ten microns appear to be the upper effective particle NMD limit for initiating tularemia in the lower respiratory tract in the monkey.

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